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Characterization of a sulfide-oxidizing biofilm developed in a packed-column reactor

Isabel Ferrera,*,¶ Olga Sánchez, Jordi Mas

Department of Genetics and Microbiology, Autonomous University of Barcelona, Bellaterra, Spain

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Summary. The potential of microbial mats to develop sulfide-oxidizing biofims was explored. A bioreactor specially designed for the treatment of sulfide-containing effluents was inoculated with a microbial-mat sample, and a complex microbial biofilm with sulfide-oxidation activity developed. The microbial composition of the biofilm was studied by pigment, microscopy, and 16S rRNA gene analyses. Purple sulfur bacteria and diatoms were observed by microscopy, chlorophyll *a* and bacteriochlorophyll *a* were detected in the pigment analysis, and high genetic diversity was found in the 16S rRNA gene library. Specialized anaerobic sulfur oxidizers (i.e., phototrophic purple and green sulfur bacteria) dominated the library. Aerobic phototrophs (diatoms) also developed and the oxygen produced allowed the growth of aerobic sulfide oxidizers, such as *Thiomicrospira*-like spp. Cyanobacteria, which are significant organisms in natural microbial mats, did not develop in the reactor but unexpected uncultured members from the Epsilonproteobacteria developed profusely. Moreover, a variety of more minor organisms, such as members of the *Cytophaga-Flavobacterium-Bacteroides* (CFB) and purple non-sulfur bacteria (*Roseospirillum* sp.), were also present. The results showed that a complex community with high genetic and metabolic diversity, including many uncultured organisms, can develop in a laboratory-scale reactor. [**Int Microbiol** 2007; 10(1):29-37]

Key words: sulfide oxidation · biofilms · microbial diversity · 16S rRNA gene

Introduction

The ability of certain microorganisms to use sulfide has been exploited for the treatment of sulfide-containing wastewaters and gas streams. Particular attention has been paid to the application of anoxygenic phototrophs (*Chlorobium* sp.) and aerobic chemolithotrophs (*Thiobacillus* sp.) [17,34]. Most of the described systems were based on the use of pure cultures growing in liquid reactors, and thus presented problems of biomass washout or instability following environmental disturbances. Recently, a fixed-biomass reactor that permits the development of complex sulfide-oxidizing biofilms was described [7,8]. The reactor operates as a sulfidostat and the control system allows highly polluted streams to be oxidized. The resident microorganisms are exposed to constant low concentrations of substrate, which avoids growth inhibition due to high sulfide concentrations.

Microbial mats have been used as natural models to study the sulfur cycle [24]. These laminated microbial communities develop in shallow marine sediments, tidal flats, and other marine and terrestrial environments, and they are composed of a wide range of metabolically active groups. Within these mats, an intense and complex sulfur cycle develops, with sulfide being produced by sulfate-reducing bacteria. Sulfide is then transformed into several different oxidized

***Corresponding author:** I. Ferrera
Dept. de Genètica i de Microbiologia, Fac. de Ciències, C3-409
Universitat Autònoma de Barcelona
08193 Bellaterra, Barcelona, Spain
Tel. +34-935813011. Fax +34-935812387
E-mail: iferrera@pdx.edu

¶Present address:
Department of Biology, Portland State University, PO Box 751
Portland, OR 97297, USA

sulfur compounds, such as elemental sulfur, polysulfides, and thiosulfate, by a diverse assembly of anoxygenic phototrophs, chemolithotrophs, and oxygenic phototrophs [5,33]. Due to their complexity and functional and genetic diversity, microbial mats have also been used in applied microbiology, for instance in the degradation of petroleum compounds [11,31]. Since microbial mats are benthic structures in which microorganisms attach to surfaces and to each other, it was hypothesized that they could serve as inoculum for the start-up of sulfide-oxidizing biofilm reactors.

In this work, we explored the potential of microbial mats for the development of effective sulfide-oxidizing biofilms. A sulfide-removal bioreactor was inoculated with a microbial mat sample from the Ebro Delta (Spain). The complex biofilm that developed was further characterized.

Materials and methods

Start-up and growth conditions. The experiment was carried out in a fixed-biomass reactor consisting of a packed column through which the sulfide-containing medium passed. The bioreactor operated as a sulfidostat, and the system maintained a constant residual sulfide concentration in the effluent. A detailed description of the reactor is presented elsewhere [7].

A synthetic medium based on the inorganic sulfide-containing medium described by Van Germerden and Beeftink [38] for anaerobic phototrophic bacteria was used in order to simulate a sulfide-polluted effluent. Sodium chloride was added at a final concentration of 3.7%. This medium contains carbonate as the only carbon source and hydrogen sulfide as electron donor, and it was prepared under anoxic conditions.

The system was inoculated with a microbial mat sample from the Ebro Delta, located in Tarragona (Spain). The sample was collected from a site called P3 (40º 40′ N, 0º 40′ E) [23]. Sampling was carried out in July of 2003, and the mats were partially inundated. A 15 × 15 cm piece of mat was excised from the sediment surface, kept at 4ºC in the dark, and transported to the laboratory. The microbial mat was homogenized in saline solution, shaken for 30 min, and then used to inoculate the bioreactor. After inoculation (day 0), biofilm development was inspected and registered daily with a digital camera.

The biofilm was exposed to a sulfide load of 1250 μM and a light irradiance of 100 μE m^{-2} s^{-1}. Samples were taken from the reactor outlet at regular intervals to check the sulfide concentration. At the end of the experiment, the packing material was removed and the biofilm was recovered by sonication and homogenized. Samples were obtained for further characterization.

Description of the inoculum. A microbial mat sample from a sand flat at the Ebro Delta was used to start up the system. At the sampling site, the microbial mats were well-developed and vertically stratified [12,23]. In the uppermost layer, diatoms were abundant. The second layer was composed mainly of the filamentous cyanobacterium *Microcoleus*, while underneath anoxygenic bacteria, especially purple sulfur bacteria, were found [14,22]. Several species of non-phototrophic bacteria, i.e., spirochetes and unidentified small rods and cocci, have been observed in such mats [21,23]. Pigment analysis of the inoculum showed the presence of chlorophyll (Chl) *a* and bacteriochlorophyll (Bchl) *a*. Phase-contrast microscopy of the sample showed that the inoculum comprised cyanobacteria, diatoms, and purple sulfur bacteria, among others (data not shown).

General analyses. Sulfide was measured by the method of Pachmayr as described by Trüper and Schlegel [37]. Pigments and protein levels were determined, and cells were observed microscopically with an epifluorescence Olympus BH microscope, as described elsewhere [8].

DNA extraction, clone library construction, and 16S rRNA gene sequencing. DNA extraction and bacterial PCR conditions were carried out as described previously [8]. The presence of Archaea was tested by using two sets of primers, 344F-GC/915R and 21F/1492R [4,29]. The bacterial PCR product was cloned with the TOPO TA cloning kit (Invitrogen) according to the manufacturer's instructions. Ninety-six putative positive colonies were picked, transferred to a multi-well plate containing Luria-Bertani medium and 7% glycerol, and stored at –80ºC. Recombinant plasmids were screened by RFLP (restriction fragment length polymorphism) analysis as described previously [8].

Forty-two clones with different band patterns were chosen for partial sequencing. Clone library coverage was calculated according to the following equation: $C=1-(n/N)$, where n is the number of unique clones and N is the total number of clones examined [26]. To determine whether the clone library was representative of the biofilm's microbial diversity, accumulation curves were constructed for the different OTUs (each different RFLP pattern) and phylotypes. Sequences sharing more than 97% similarity were grouped as the same phylotype.

Double-stranded plasmids were extracted with a QIAprep Kit (QIAgen) from selected clones. Sequencing reactions were done by Sistemas Genómicos (Spain) [http://www.sistemasgenomicos.com] with the primer 27F and run in an ABI PRISM 377 XL lane sequencer. Sequences were subjected to a BLAST search [2] to obtain an initial indication of the phylogenetic affiliation, and to the CHECK-CHIMERA program from RDP [20] to determine potential chimeric artifacts. Sequences were aligned with the automatic tool of ARB and then manually adjusted according to the secondary structure constraints of 16S rRNA [19].

The optimized maximum parsimony tree obtained from the ARB software and derived from complete sequence data was used to add the partial clone sequences from this study according to the "Quick add using parsimony" tool, which does not affect the initial tree topology. Clone sequences were approximately 700 nucleotides in length (see Table 1 for exact sequence length) and corresponded approximately to positions 50–750 (*E. coli* 16S rRNA gene sequence numbering). The resulting tree was trimmed to save space and only the closest relatives to the clone sequences of interest were retained. Similarity matrices between similar sequences were calculated with an ARB tool.

The 16S rRNA gene sequences obtained from the biofilm were submitted to the EMBL database under accession numbers AJ627979 to AJ628015.

Results

Biofilm development. Following inoculation, cell attachment occurred slowly, so that the entire column was completely colonized only after 10 days (Fig. 1). The appearance of the column changed progressively, until finally a complex multicolored biofilm had developed. The sulfide oxidation measurements agreed with the temporal development of the biofilm. The initial concentration of sulfide in the reactor decreased progressively, to 50 μM during the first 6 days, and then remained constant until the end of the experiment. During these first 6 days, no fresh medium was pumped into the reactor, and therefore no effluent was produced. From then on, the

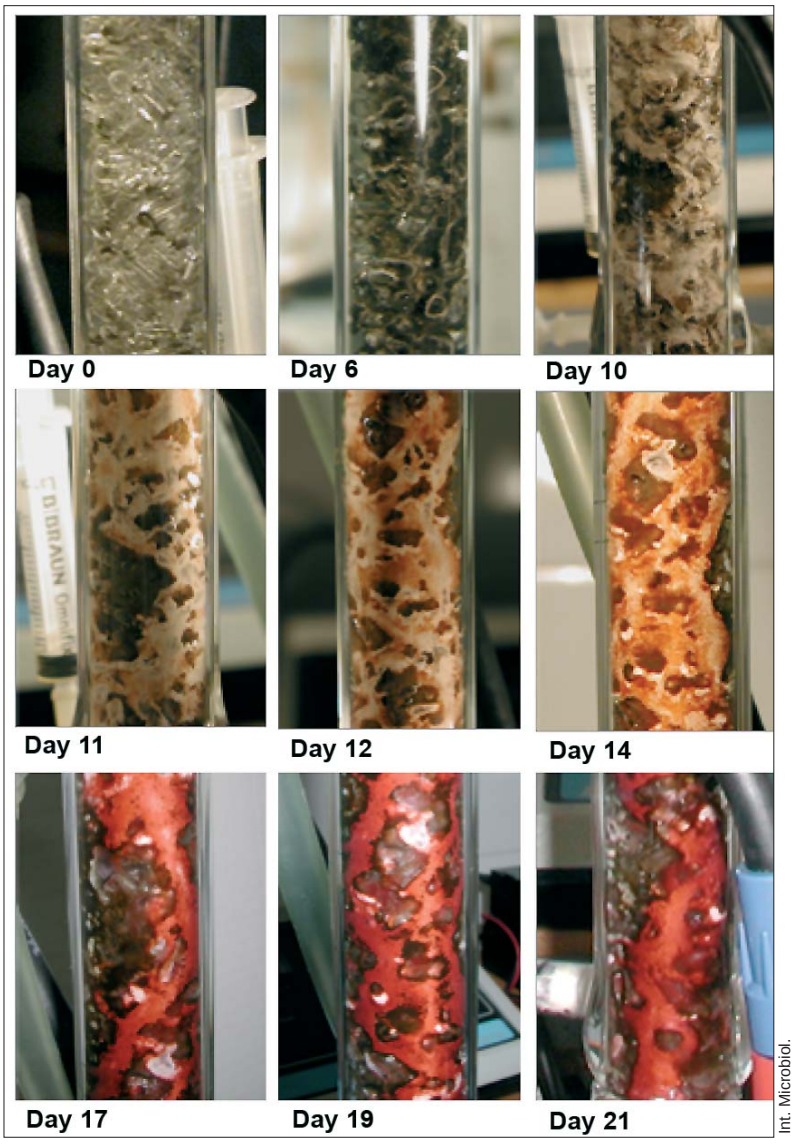

Fig. 1. Development of a sulfide-oxidizing biofilm. Significant rates of sulfide-oxidation began after day 6.

pump speed was increased gradually and the sulfide oxidation rate reached 158 μmol h^{-1}. Under these conditions, the sulfide concentration was reduced by 96%. After a run-time of 21 days, and once the oxidation rate stabilized, the experiment was terminated and the biofilm was characterized.

Characterization of the biofilm. Phase-contrast and epifluorescence microscopy observations showed the presence of several different morphotypes, including diatoms and purple sulfur bacteria. However, many cells did not show any characteristic morphological traits and therefore could not be identified. Biomass recovered from the column totaled 431 mg of protein, which corresponded to a density of 1.33 mg protein cm^{-2}. Pigment analysis indicated the presence of Chl *a* (10.8 μg cm^{-2}) and Bchl *a* (21.5 μg cm^{-2}).

Clone library construction. Bacterial PCR products were used to construct a clone library and 37 different sequences were obtained. The retrieved sequences, their frequency in the library, and their closest relatives are listed in Table 1. Sequences sharing more than 97% similarity were grouped, which decreased the number of phylotypes to 26. The coverage of the library was 86.5%. Accumulation curves indicated that most of the diversity in the library was recovered (data not shown). Archaea were not detected in the biofilm by PCR amplification with two different primer sets, various DNA template levels, and a range of PCR cycling conditions.

16S rRNA gene sequencing and phylogenetic analysis. The partial 16S rRNA gene sequences recovered were included in the two phylogenetic trees (Figs. 2 and 3).

Table 1. Phylogenetic affiliation of clones to the closest match in Genbank (n, frequency; nt, sequence length)

Group	Name	n	nt	Closest match	Acc. No.	Similarity
Alphaproteobacteria	2B4	1	692	*Rhodovulum adriaticum*	D16418	100
	1E5	1	674	*Erythrobacter litoralis*	AF465836	99.6
	1G1	1	669	*Roseospirillum parvum*	AJ011919	99.5
	2E12	2	681	*Roseospirillum parvum*	AJ011919	99.5
	2H6	1	686	*Roseospirillum parvum*	AJ011919	99.5
	1B9	8	693	*Roseospirillum parvum*	AJ011919	99.2
Gammaproteobacteria	1F4	1	648	*Marichromatium purpuratum*	AF294031	99.7
	3A9	1	700	*Marichromatium purpuratum*	AF294031	99.7
	2G6	3	715	*Marichromatium purpuratum*	AF294031	99.6
	1A10	3	621	*Marichromatium purpuratum*	AF294030	99.5
	2G3	1	709	*Marichromatium purpuratum*	AF294031	99.4
	1B2	69	695	*Marichromatium purpuratum*	AF294031	98.6
	1G4	1	690	*Thiorhodococcus drewsii*	AF525306	99.4
	2C9	2	697	*Thiomicrospira chilensis*	AF013975	99.6
	2E11	1	675	*Thiomicrospira kuenenii*	AF013978	95.4
	1C3	1	667	*Vibrio* sp.	AF064637	99.0
	2E10	2	651	*Vibrio* sp.	AY374383	98.9
	1D3	1	673	Uncultured gammaproteobacterium	AF453551	94.7
	1F10	1	656	Uncultured gammaproteoacterium	AF453551	94.7
	1G5	1	611	*Escarpia spicata* endosymbiont	AF165908	92.0
Epsilonproteobacteria	1B6	1	654	Uncultured *Sulfurospirillum*	AF513952	97.2
	1C6	26	639	Uncultured epsilonproteobacterium	AJ441205	96.9
	1F5	1	656	Uncultured epsilonproteobacterium	AJ441205	96.0
	1B8	1	630	Uncultured epsilonproteobacterium	AJ575993	95.2
	2A6	5	642	Uncultured epsilonproteobacterium	AJ575993	95.2
	1D10	4	660	Uncultured epsilonproteobacterium	AJ575993	91.9
	CFB	1	648	*Marinilabilia salmonicolor*	M62423	96.0
	1G6	1	688	*Marinilabilia salmonicolor*	M62422	94.8
	1A5	1	672	*Marinilabilia salmonicolor*	M62422	94.5
	1A4	2	645	Uncultured Bacteroidetes	AJ347762	94.3
	1D5	6	690	Uncultured bacterium	AJ548901	91.3
	2A10	1	643	Bacteria from anoxic bulk soil	AJ229236	90.5
	2F2	1	616	Bacteria from anoxic bulk soil	AJ229236	90.1
Chlorobi	1B5	8	650	Chlorobiaceae bacterium	AF513460	98.6
Plastids	1D12	7	697	*Haslea salstonica*	AF514854	98.2
Not-determined *Bacteria*	2G8	1	663	Uncultured bacterium	AF371929	88.8
	1F8	1	676	Uncultured bacterium clone SJA-101	AJ009480	88.3

Different phylogenetic groups previously identified by microscopy were retrieved, such as green algae (diatoms) and Gammaproteobacteria (i.e., Chromatiaceae). In addition, Alpha- and Epsilonproteobacteria and the *Cytophaga-Flavobacterium-Bacteroides* (CFB) group, which are difficult to ascertain by microscopy, were also detected. Gammaproteobacteria dominated the library (52% of total clones), mostly with representatives of *Chromatium* and relatives (up to 46%). Epsilonproteobacteria were also dominant in the library (22% of total clones) while Alphaproteobacteria were less abundant (8%). The remaining clones belonged to chloroplasts from algae (4%), Chlorobi (5%), and the CFB (8%). One percent of the clones was not affiliated to a significant extent with any phylogentetic group.

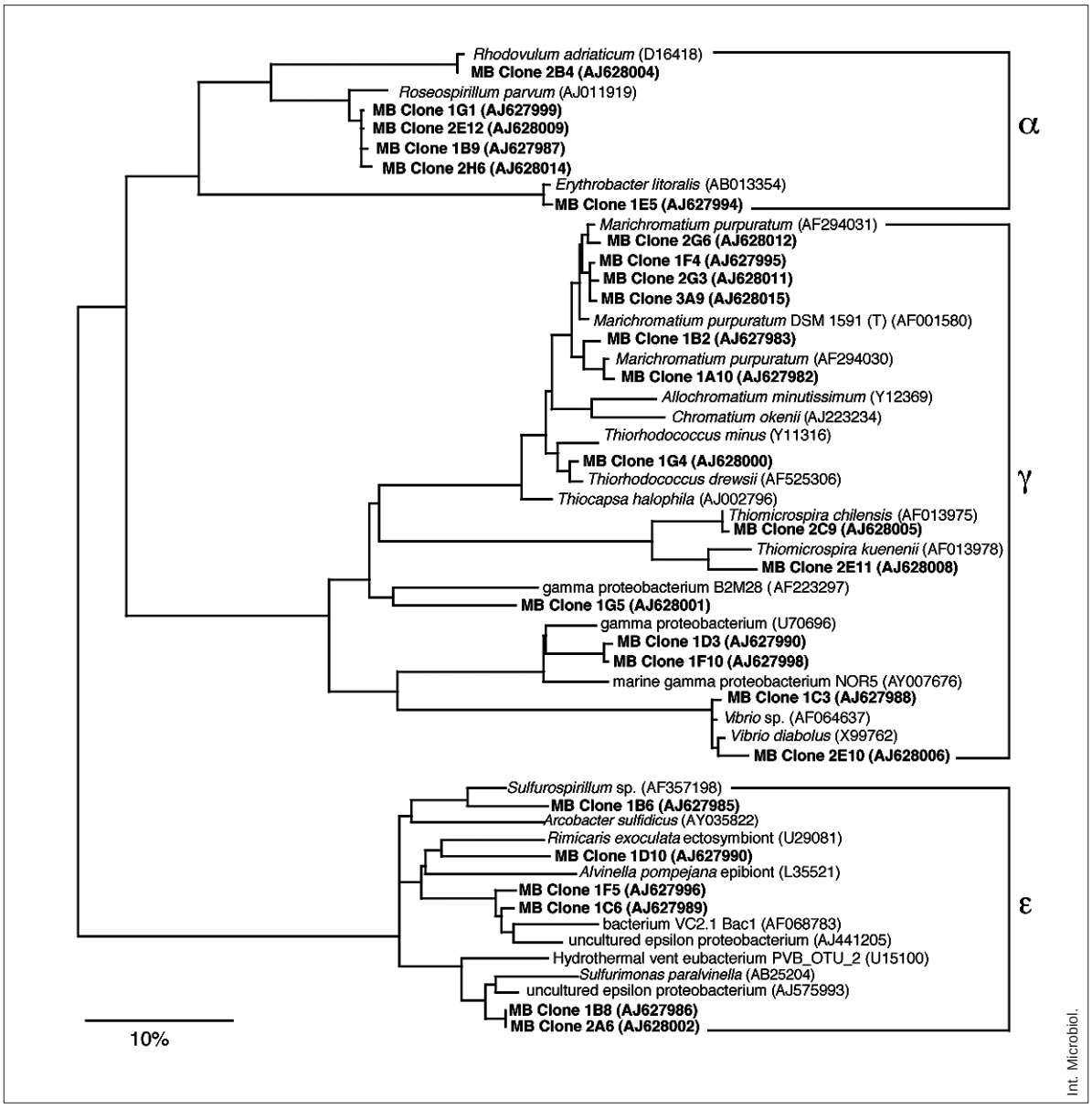

Fig. 2. Phylogenetic tree including sequences recovered from the biofilm that were affiliated witih Proteobacteria (sequence accession numbers indicated).

Most of the clones were highly related to previously described sequences in the GenBank database, and 63% of the clones had more than 97% similarity to cultured species. All clones related to purple sulfur bacteria were affiliated with *Marichromatium purpuratum* while other Gammaproteobacteria, present but less abundant, were affiliated with cultured strains of the genera *Thiomicrospira* and *Vibrio*. All clones affiliated with the Chlorobiaceae were highly related to *Prosthecochloris aestuarii*. Among the alpha subclass, most clones were related to the so-called phototrophic purple Alphaproteobacteria, in particular to *Roseospirillum parvum*, which was originally isolated from a marine microbial mat [10]. Chloroplasts from algae were also present in the bacterial clone library (4%). In agreement with microscopy observations, sequences related to a diatom (97.4% similarity) were detected. Sequences belonging to the CFB cluster accounted for 8% of the clones, with some being related to the marine species *Marinilabilia salmonicolor*. Most of the sequences belonging to the Epsilonproteobacteria were affiliated with uncultured organisms from hydrothermal deep-sea vents and only one sequence matched that of *Sulfurospirillum* sp.

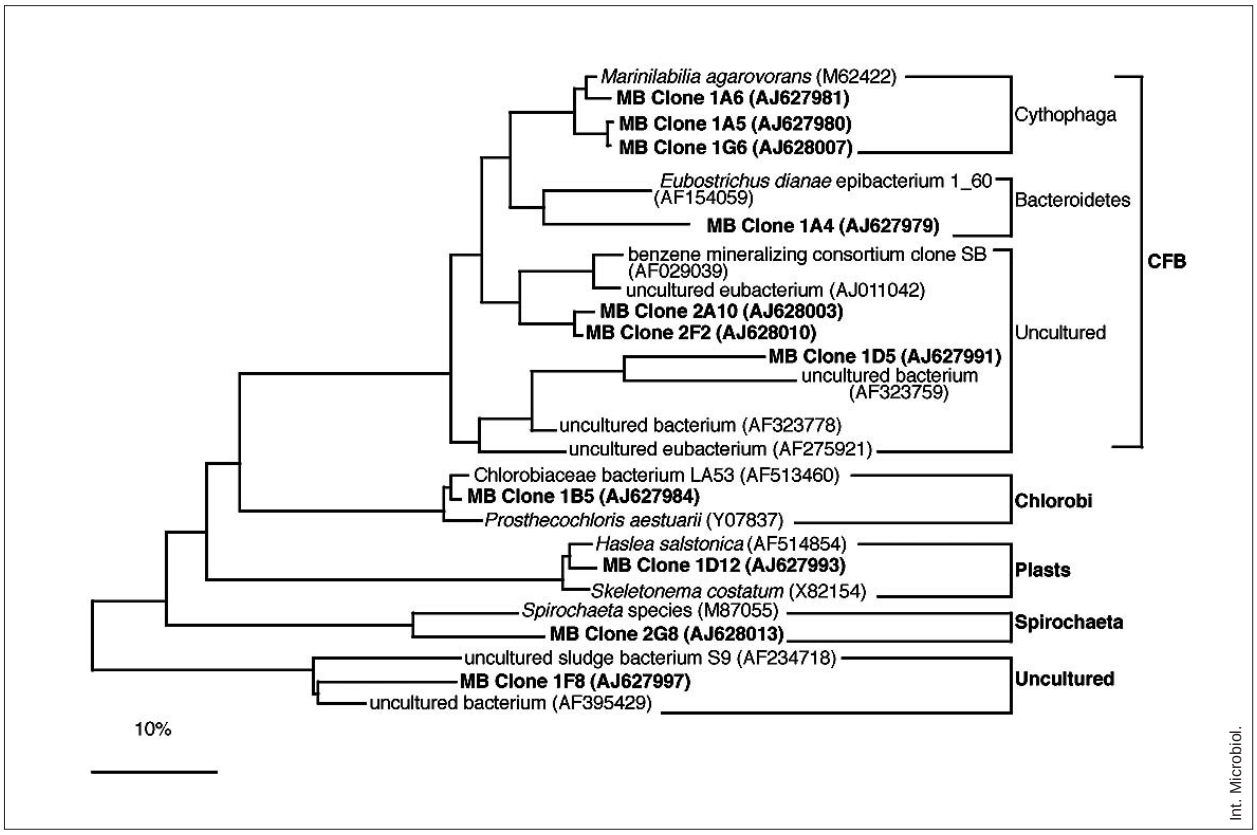

Fig. 3. Phylogenetic tree including sequences recovered from the biofilm and affiliated with the CFB group, Chlorobi, plastids, and other bacteria (sequence accession numbers indicated).

Phylogenetic analyses also revealed that some of the retrieved sequences showed a considerable degree of microdiversity [9], especially those clones related to *Roseospirillum parvum* and *Marichromatium purpuratum* (see phylogenetic tree, Fig. 2). Similarity matrices between such similar sequences showed that four *Roseospirillum-parvum*-like sequences and three *Marichromatium purpuratum*-like sequences were, on average, 99.6 and 99.3% similar to each other, respectively.

Discussion

A complex biofilm aimed at the oxidation of sulfide-containing effluents was developed after inoculation of a bench-scale reactor with a microbial-mat sample. Some organisms present in the biofilm could be identified by microscopy (purple sulfur bacteria and diatoms) but most of them could not. The latter were instead identified by 16S rRNA gene sequencing. Clone library analyses are based on PCR analyses, which could present quantitative biases [28,40], and thus should be understood within that context. Nonetheless, although the relative abundance of each group in the library probably differed from its real contribution, in our study, the presence of some groups recovered by cloning was confirmed by microscopy and pigment analysis (i.e., purple sulfur bacteria and green algae). In the clone library, the majority (63%) of sequences recovered had 16S rRNA gene similarities at the species level with cultured species (>97%) [32]. Clearly, it is not possible to infer a functional trait based merely on 16S rRNA sequence similarity; however, because the sequences described here were closely related to well-characterized cultured strains and the prevailing environmental conditions within the bioreactor were well-established, putative functions could be assigned to the microorganisms represented by 16S rRNA sequences. As expected, most of the microorganisms were involved in the sulfur cycle. Specialized anaerobic sulfur oxidizers carrying out anoxygenic photosynthesis, such as purple and green sulfur bacteria, dominated the biofilm. Both microorganisms are typically found in benthic-illuminated environments containing sulfide, such as microbial mats. In particular, purple sulfur bacteria that developed in the bioreactor were affiliated with *Marichromatium purpuratum* at the species level (>99% sim-

ilarity in 16S rRNA gene). This species is frequently found in association with sponges [15] rather than in suspension, and this ability may facilitate its profuse attached growth. Although some species of green sulfur bacteria have been isolated from microbial mats, usually purple bacteria dominate mats to the exclusion of green species, and they are therefore rarely abundant in such ecosystems. Green sulfur bacteria highly related to *Prosthecochloris aestuarii* were present in the biofilm but, as occurs in nature, at a lower level than purple sulfur bacteria, very likely because the former are strict anaerobes whereas the latter tolerate low oxygen concentrations, and oxygen producers were also present in the biofilm.

In addition to sulfur bacteria, diatoms were another important phototrophic group identified in the biofilm. In the bioreactor, since there was no aeration, they would represent a source of oxygen produced by photosynthesis, which could be used by aerobic organisms. Diatoms are usually found in the upper layer of microbial mats together with cyanobacteria. In Ebro Delta microbial mats, the filamentous cyanobacteria *Microcoleus* and *Lyngbya* have been reported as the most abundant oxygenic phototrophs [12,23]. Some cyanobacteria are highly tolerant to sulfide and can even operate photosystem I using sulfide as an electron donor for photosynthesis. Although their development in the column was therefore expected, algae developed instead. Competition for light or nitrogen limitation could explain their absence, but the exact reason remains unclear. However, from an applied point of view, the two organisms would have the same function in the biofilm—oxygen production through oxygenic photosynthesis—and therefore facilitate the development of other aerobes. Thus, although taxonomically the structure of the biofilm was not identical to a microbial mat, phototrophic metabolism was represented in the biofilm, in particular by aerobic (diatoms) and anaerobic (purple and green sulfur bacteria) phototrophs.

Other less abundant Gammaproteobacteria included those affiliated with bacteria belonging to the genera *Thiomicrospira*, which is able to chemolithotrophically oxidize reduced sulfur compounds [30], and *Vibrio,* which is a heterotrophic bacteria [6]. Among the Alphaproteobacteria, most clones were related to the purple nonsulfur bacteria *Roseospirillum parvum*. This group shows high metabolic versatility in that it is able to carry out anoxygenic photosynthesis using CO_2 or organic matter as carbon source and organic matter or reduced sulfur compounds as electron donors [13]. In nature, these bacteria are often found together with purple sulfur bacteria. While freshwater isolates have very low tolerance of sulfide, most marine species are resistant to higher concentrations and can even use sulfide and thiosulfate as photosynthetic electron donors**.** Obviously, in our bioreactor, such microorganisms tolerated sulfide and may have grown autotrophically using sulfide as electron donor.

Cytophaga-Flavobacterium-Bacteroides-related organisms also developed in the biofilm. These bacteria are abundant in many organic-material-rich habitats and play a major role in the turnover of organic matter in such environments [16]. Within this group, aerobic, microaerophilic, and anaerobic species are found, and most cultured CFB are chemoorganotrophs. In the past, their presence in sediments could not be investigated because of the lack of appropriate techniques for their identification. However, recent studies have revealed the importance of CFB in microbial mat ecosystems [25]. Most 16S rRNA gene sequences from the CFB cluster included in databases belong to uncultured organisms. Some of the sequences determined in the present study were related to the marine species *Marinilabilia salmonicolor* (synonym *Cytophaga salmonicolor*), which like most *Cytophaga* species is able to grow under microaerophilic conditions [27]. However, other sequences were indeed related to uncultured organisms.

The most surprising finding was that a significant number of sequences were related to uncultured Epsilonproteobacteria. Several recent studies have demonstrated the presence and dominance of Epsilonproteobacteria at deep-sea hydrothermal vents [1,29,36]. Moreover, Epsilonproteobacteria have also been detected and/or isolated from deep subsurface sediments, oil fields, activated sludge, marine snow, coastal sediments, and in the oxic/anoxic chemocline from the Black Sea [18,39,41]. These organisms are considered to be key players in the cycling of carbon, nitrogen, and sulfur in sulfur-rich environments [3]. Although there are many epsilonproteobacterial sequences in databases, only a limited number of isolates have been obtained. There is evidence indicating their use of the reverse TCA cycle for chemolithoautotrophy [3,35]. Autotrophic growth on sulfide, elemental sulfur, thiosulfate, and hydrogen as electron donors under anoxic or microaerophilic conditions has been described [3,35].

Although the Epsilonproteobacteria have never been reported as important members of mesophilic microbial mats, the environmental conditions in our biofilm appear to have been optimal for their growth. The sequences identified here were related to hydrothermal deep-sea bacteria. Unfortunately, conclusions regarding the metabolic activities of these microorganisms in the biofilm must be based on only a few clones. However, taking into account the environmental conditions within the reactor, they may be involved in sulfide oxidation. Bioreactors such as the one described here

could be very useful for further metabolic studies or even for the isolation of these bacteria.

The data obtained in this work were compared to those from a freshwater biofilm used in a similar experiment [8]. Biomass and oxidation rates were comparable, although slightly higher values were obtained in the freshwater biofilm (1.93 mg protein cm^{-2} and 200 μmol h^{-1}). Both biofilms presented high-species richness and many different groups were represented. However, phylogenetically these biofilms were different. For instance, phototrophic sulfur bacteria in the freswater biofilm were *Thiocapsa* sp. whereas here *Marichromatium*, *Thiorhodococcus* and *Prothecochloris* were found. No epsilonproteobacterial sequences were obtained in the previous experiment, but other sulfur-oxidizers were detected (*Thiothrix, Thiobacillus*). Nonetheless, from functional and applied points of view, the two biofilms were efficient and comparable. Phototrophic sulfur bacteria, purple non-sulfur bacteria, aerobic sulfur oxidizers, oxygenic phototrophs, and heterotrophs were present in both.

In summary, a marine microbial mat sample was found to yield a biofilm capable of the steady-state removal of sulfide. The biofilm was characterized by high genetic diversity and the coexistence of metabolically diverse groups. Cell immobilization in the reactor facilitated the presence of organisms that constituted only a minor part of the original community and that would have been washed out in liquid systems. This contributed to the diversity and complexity of the assemblage, in which a wide array of metabolic pathways was available. Many sequences were affiliated with uncultured organisms, indicating that a bioreactor provides a growth environment very different from the conditions traditionally used for isolating new organisms.

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References

1. Alain K, Olagnon M, Desbruyères D, Pagé A, Barbier G, Juniper SK, Quérellou J, Cambon-Bonavita MA (2002) Phylogenetic characterization of the bacterial assemblage associated with mucous secretions of the hydrothermal vent polychaete *Paralvinella palmiformi*. FEMS Microbiol Ecol 42:463-476
2. Altschul SF, Madden TL, Schäffer AA, et al. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25:3389-3402
3. Campbell BJ, Campbell BJ, Engel AS, Porter ML, Takai K (2006) The versatile ε-proteobacteria: key players in sulphidic habitats. Nat Rev Microbiol. 4:458-468
4. Casamayor EO, Schäfer H, Bañeras L, Pedrós-Alió C, Muyzer G (2000) Identification of and spatio-temporal differences between microbial assemblages from two neighboring sulfurous lakes: comparison by microscopy and denaturing gradient gel electrophoresis. Appl Environ Microbiol 66:499-508
5. Caumette P, Matheron R, Raymond N, Relexans JC (1994) Microbial mats in the hypersaline ponds of Mediterranean salterns (Salins-de-Giraud, France). FEMS Microbiol Ecol 13:273-286
6. Farmer III JJ, Hickman-Brenner FW (2006) The genera *Vibrio* and *Photobacterium.* In: Dworkin M, et al. (eds) The prokaryotes. Springer, New York, Vol 6, Part 3, pp 508-563
7. Ferrera I, Sánchez O, Mas J (2004) A new non-aerated illuminated packed-column reactor for the development of sulfide-oxidizing biofilms. Appl Microbiol Biotech 64:659-664
8. Ferrera I, Massana R, Casamayor EO, Balagué V, Sánchez O, Pedrós-Alió C, Mas J (2004) High-diversity biofilm for the oxidation of sulfide-containing effluents. Appl Microbiol Biotech 64:726-734
9. Furhman JA, Campbell L (1998) Microbial microdiversity. Nature 393:410-411
10. Glaeser J, Overmann J (1999) Selective enrichment and characterization of *Roseospirillum parvum*, gen. nov. and sp. nov., a new purple nonsulfur bacterium with unusual light absorption properties. Arch Microbiol 171:405-416
11. Grötzschel S, Köster J, Abed RMM, de Beer D (2002) Degradation of petroleum model compounds immobilized on clay by a hypersaline microbial mat. Biodegradation 13:273-283
12. Guerrero R, Urmeneta J, Rampone G (1993) Distribution types of microbial mats at the Ebro Delta, Spain. BioSystems 31:135-144
13. Imhoff JF (2006) The phototrophic Alpha-Proteobacteria. In: Dworkin M, et al. (eds) The prokaryotes. Springer, New York, Vol 5, Part 1, pp 41-64
14. Imhoff JF (2006) The Chromatiaceae. In: Dworkin M, et al. (eds) The prokaryotes, Springer, New York, Vol. 7, Part 3, pp 846-873
15. Imhoff JF, Trüper HG (1976) Marine sponges as habitats of anaerobic phototrophic bacteria. Microb Ecol 3:1-9
16. Kirchman DL (2002) The ecology of *Cytophaga-Flavobacteria* in aquatic environments. FEMS Microbiol Ecol 39:91-100
17. Kobayashi HA, Stenstrom M, Mah RA (1983) Use of photosynthetic bacteria for hydrogen sulfide removal from anaerobic waste treatment effluent. Water Res 17:579-587
18. Kodama Y, Watanabe K (2003) Isolation and characterization of a sulfur-oxidizing chemolithotroph growing on crude oil under anaerobic conditions. Appl Environ Microbiol 69:107-112
19. Ludwig W, Strunk, O, Klugbauer S, et al. (1998) Bacterial phylogeny based on comparative sequence analysis. Electrophoresis 19:554-568
20. Maidak BL, Cole JR, Lilburn TG, et al. (2000) The RDP (Ribosomal Database Project) continues. Nucleic Acids Res 28:73-174
21. Margulis L, Ashen JB, Solé M, Guerrero R (1993) Composite, large spirochetes from microbial mats: spirochete structure review. Proc Natl Acad Sci USA 90:6966-6970
22. Martínez-Alonso M, Van Bleijswijk J, Gaju N, Muyzer G (2005) Diversity of anoxygenic phototrophic sulfur bacteria in the microbial mats of the Ebro Delta: a combined morphological and molecular approach. FEMS Microbiol Ecol 52:339-350.
23. Mir J, Martínez-Alonso M, Esteve I, Guerrero R (1991) Vertical stratification and microbial assemblage of a microbial mat in the Ebro Delta (Spain). FEMS Microbiol Ecol 86:59-68
24. Mir J, Martínez-Alonso M, Caumette P, Guerrero R, Esteve I (2002) Sulfide fluxes in a microbial mat from the Ebro Delta, Spain. Int Microbiol 5:133-138
25. Mouné S, Caumette P, Matheron R, Willison JC (2003) Molecular sequence analysis of prokaryotic diversity in the anoxic sediments underlying cyanobacterial mats of two hypersaline ponds in Mediterranean salterns. FEMS Microbiol Ecol 44:117-130
26. Ravenschlag K, Sahm K, Pernthaler J, Amann R (1999) High bacterial diversity in permanently cold marine sediments. Appl Environ Microbiol 65:3982-3989

27. Reichenbach H (2006) The Order Cytophagales. In: Dworkin M, et al. (eds) The prokaryotes. Springer, New York, Vol 7, Part 6, pp 549-590
28. Reysenbach AL, Giver LJ, Wickham GS, Pace NR (1992) Differential amplification of rRNA genes by polymerase chain reaction. Appl Environ Microbiol 58:3417-3418
29. Reysenbach AL, Longnecker K, Kirshtein J (2000) Novel bacterial and archaeal lineages from an in situ growth chamber deployed at a Mid-Atlantic Ridge hydrothermal vent. Appl Environ Microbiol 66:3798-3806
30. Robertson LA, Kuenen JG (2006) The colorless sulfur bacteria. In: Dworkin M, et al. (eds) The prokaryotes. Springer, New York, Vol 2, Part 1, pp 985-1011
31. Sánchez O, Ferrera I, Vigués N, García de Oteyza T, Grimalt J, Mas J (2006) Presence of opportunistic oil-degrading microorganisms operating at the initial steps of oil extraction and handling. Int Microbiol 9:119-124
32. Stackebrandt E, Goebel BM (1994) Taxonomic note: a place for DNA:DNA reassociation and 16S rRNA sequence analysis in the present definition in bacteriology. Int J Syst Bacteriol 44:846-849
33. Stal LJ (1991) Microbial mats in coastal environments. In: Stal LJ, Caumette P (eds) Microbial mats: structure, development and environmental significance. NATO Series, Springer-Verlag, Heidelberg, Vol G35, pp 21-32
34. Sublette KL, Sylvester ND (1987) Oxidation of hydrogen sulfide by continuous cultures of *Thiobacillus denitrificans*. Biotechnol Bioeng 29:753-758
35. Takai K, Campbell BJ, Cary SC, et al. (2005) Enzymatic and genetic characterization of carbon and energy metabolisms by deep-sea hydrothermal chemolithoautotrophic isolates of *Epsilonproteobacteria*. Appl Environ Microbiol 71:7310-7320
36. Takai K, Suzuki M, Nakagawa S, Miyazaki M, Suzuki Y, Inagaki F, Horikoshi K (2006) *Sulfurimonas paralvinellae* sp. nov., a novel mesophilic, hydrogen- and sulfur-oxidizing chemolithoautotroph within the *Epsilonproteobacteria* isolated from a deep-sea hydrothermal vent polychaete nest, reclassification of *Thiomicrospira denitrificans* as *Sulfurimonas denitrificans* comb. nov. and emended description of the genus *Sulfurimonas*. Int J Syst Evol Microbiol 56:1725-1733
37. Trüper HG, Schlegel HG (1964) Sulphur metabolism in Thiorhodaceae. 1. Quantitative measurements on growing cells of *Chromatium okenii*. Ant Leeuw 30:225-238
38. Van Gemerden H, Beeftink HH (1978) Specific rates of substrate oxidation and product formation in autotrophically growing *Chromatium vinosum* cultures. Arch Microbiol 119:932-943
39. Vetriani C, Tran HV, Kerkhof LJ (2003) Fingerprinting microbial assemblage from the oxic/anoxic chemocline of the Black Sea. Appl Environ Microbiol 69:6481-6488
40. Von Wintzingerode FV, Goebel UB, Stackebrandt E (1997) Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS Microbiol Rev 21:213-22
41. Wirsen CO, Sievert SM, Cavanaugh CM, Molyneaux SJ, Ahmad A, Taylor LT, DeLong EF, Taylor CD (2002) Characterization of an autotrophic sulfide-oxidizing marine *Arcobacter* sp. that produces filamentous sulfur. Appl Environ Microbiol 68:316-312